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# Antibodies to Actin in Autoimmune Neutropenia

By Kip R. Hartman, Maryrita K. Mallet, Jayasree Nath, and Daniel G. Wright

In an effort to characterize the cellular antigens recognized by anti-neutrophil antibodies in autoimmune neutropenia, we studied sera, purified immunoglobulin G (IgG) and isolated F(ab')<sub>2</sub> from 70 neutropenic patients suspected of this diagnosis. Anti-neutrophil antibodies were found in the sera of 36 of these patients by either <sup>125</sup>I-staph A binding or immunofluorescence cytometric techniques that detected increased binding of patients' IgG to normal neutrophils. Anti-neutrophil antibody positive sera were then evaluated for specific binding to electrophoretically separated neutrophil membrane-associated proteins by immunoblotting. A 43-Kd protein was consistently identified by eight anti-neutrophil antibody positive sera. The specificity of binding to this protein was confirmed with affinity purified IgG and F(ab')<sub>2</sub> fragments prepared from these sera. Sera from 20 healthy normal controls and from 22 non-neutropenic, anti-neutrophil antibody negative rheumatoid arthritis patients failed to bind this protein. Separate studies identified

the 43-Kd protein as actin. Purified *Acanthamoeba* actin comigrated with the protein and was specifically bound by anti-neutrophil antibody positive IgG. Moreover, two actin-specific monoclonal antibodies bound to the 43-Kd membrane-associated protein in immunoblots. In addition, a rabbit anti-actin antiserum not only bound to this same 43-Kd protein but also expressed anti-neutrophil antibody activity against normal human neutrophils, as did purified human anti-actin IgG prepared by affinity chromatography from the serum of one of the index patients. These studies indicate that the anti-neutrophil antibodies of certain patients with autoimmune neutropenia include autoantibodies specific for actin. The molecules on the surface of neutrophils, which have actin-like antigenic epitopes and are recognized by these anti-actin antibodies, remain to be characterized.

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ANTI-NEUTROPHIL antibodies have been detected in a variety of clinical conditions associated with neutropenia, such as rheumatoid arthritis, systemic lupus erythematosus, chronic active hepatitis, immune thrombocytopenic purpura, human immunodeficiency virus disease, and drug reactions.<sup>1-4</sup> Anti-neutrophil antibodies have also been detected in adults and children with idiopathic neutropenias not associated with an identifiable underlying disease. In certain cases, the degree of neutropenia has been correlated with the titer of anti-neutrophil antibodies, implying an etiologic role for these antibodies in the neutropenia.<sup>5,6</sup> The maturational specificity of anti-neutrophil antibodies has also been related to the severity of neutropenia, with antibodies that bind to immature myeloid cells being associated with the most severe neutropenias.<sup>7</sup>

A variety of methods for detecting anti-neutrophil antibodies have been developed, and neutrophil antigens have been classified using serologic techniques.<sup>8</sup> In addition, anti-neutrophil antibodies have been reported in some cases to alter neutrophil function, including superoxide generation,<sup>9,10</sup> chemotaxis,<sup>11</sup> and deformability.<sup>12</sup> However, very little is known about the antigenic specificities of naturally occurring anti-neutrophil autoantibodies that are associated with neutropenia.

To characterize the antigens recognized by anti-neutrophil autoantibodies, we studied sera, purified immunoglobulin G

(IgG), and isolated F(ab')<sub>2</sub> fragments from patients with suspected autoimmune neutropenia, using an immunoblot technique applied to electrophoretically separated neutrophil membrane proteins. In a subset of patients with neutropenia and anti-neutrophil antibodies, we detected specific IgG binding to a 43-Kd neutrophil membrane-associated protein that was identified as actin. Furthermore, affinity-purified anti-actin autoantibodies isolated from the serum of one of these patients was found to bind specifically to intact neutrophils, indicating that autoantibodies with anti-actin specificities recovered from patients with autoimmune neutropenia may contribute to the anti-neutrophil antibody reactivities of these sera.

## MATERIALS AND METHODS

**Human subjects and serum samples.** Sera screened for anti-neutrophil antibodies were collected from 70 patients with suspected autoimmune neutropenia, referred from the adult and pediatric services of Walter Reed Army Medical Center (WRAMC), Washington, DC, and elsewhere (see Acknowledgment). Control sera were collected from 20 healthy normal volunteers and 22 non-neutropenic patients with rheumatoid factor-positive rheumatoid arthritis. Control sera of blood types A, B, AB, and O, and/or pooled ABO sera, were tested with each assay. Sera were heat-inactivated at 56°C for 30 minutes and frozen at -70°C before use. Serum samples were obtained with the consent of the patients and their referring physicians according to a protocol approved by the WRAMC Human Use Committee.

**Detection of anti-neutrophil antibodies.** Heparinized whole blood, or neutrophil-enriched leukapheresis preparations, were collected from normal volunteer donors. Purified neutrophils were prepared by Ficoll/hypaque centrifugation, dextran sedimentation, and hypotonic lysis of residual red cells.<sup>13</sup> Neutrophils were more than 97% viable by trypan blue exclusion, and more than 95% free of other cell types. Neutrophils for immunofluorescence assays were suspended in phosphate buffered saline (PBS) with 2% fetal calf serum and 0.1% sodium azide; neutrophils for radiometric assays were preserved with 1% paraformaldehyde in PBS before use.

The radiometric anti-neutrophil assay was adapted from a method described by Blumfelder and Logue.<sup>14</sup> Neutrophils were incubated with test or control sera, washed twice, then incubated with radioiodinated [<sup>125</sup>I] staphylococcal protein A (SPA) (Amersham,

*From the Department of Hematology, Walter Reed Army Institute of Research, Washington, DC.*

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*Address reprint requests to Kip R. Hartman, MD, Department of Hematology, Walter Reed Army Institute of Research, Washington, DC 20307-5100.*

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Arlington Heights, IL). Cells were then spun through phthalate oil (dibutyl phthalate [Sigma, St Louis, MO]; bis[2-ethylhexyl]-phthalate [Eastman Kodak Co, Rochester, NY], 1.5:1), and radioactivity of the cell pellet was determined in a gamma counter. Results were expressed as fg SPA bound per cell, and positive results were defined as greater than a 95% confidence interval calculated for the mean values of 20 normal controls.

For the immunofluorescence assay, fluorescein isothiocyanate (FITC) goat anti-human IgG and FITC goat anti-mouse IgG (Fab fragments, heavy- and light-chain specific) were obtained from Cappel Laboratories, Cooper Biomedical Inc, Malvern, PA. Anti-neutrophil murine monoclonals, which served as positive control Igs, were obtained from the following sources: OKM1 from Ortho Diagnostic Systems Inc, Raritan, NJ; anti-human Leu-M1 from Becton Dickinson Monoclonals Center Inc, Mountain View, CA; anti-human Mol and isotype specific murine control Igs from Coulter Immunology, Hialeah, FL. Rabbit anti-actin antiserum and FITC goat anti-rabbit IgG were obtained from ICN Immunobiologicals, Naperville, IL. Cells were analyzed on a Coulter EPICS V System integrated with a Coulter MDADS Computer (Coulter Electronics, Hialeah, FL).

In the immunofluorescence assay, neutrophils were incubated with test or control sera, diluted 1:20 for 30 minutes at 4°C, washed twice, then incubated with a fluoresceinated second antibody, and washed twice again. Cells were preserved in 1% paraformaldehyde before fluorescence activated cell analysis. Neutrophils labeled with test and control human sera were incubated with FITC goat anti-human IgG (Fab fragment) as the second antibody. Cells labeled with murine monoclonal antibodies (MoAbs) and murine isotype control Igs were incubated with FITC goat anti-mouse Ig (Fab fragment) as the second antibody. Cells labeled with rabbit anti-actin antiserum and normal rabbit control serum were incubated with FITC goat anti-rabbit IgG as the second antibody.

Results were expressed in terms of computer-generated histograms of cell number versus fluorescence. Fluorescence flow cytometry used a wavelength of 488 nm. Cells were analyzed by log-integrated green fluorescence (LIGFL) with the forward-angle light scatter gated on the neutrophil population. Ten thousand cells were counted in each assay; all samples were run in duplicate. The high voltage was set such that the LIGFL histogram for the serum-free control was gaussian, with a peak at channel  $35 \pm 5$  (mean  $\pm$  SD). The MDADS System has a scale of 256 channels on the x-axis. Ranges of fluorescence for a cell population were calculated by excluding the lower and upper 1% of fluorescent events. Twenty normal sera were assayed and found to be less than or equal to a pooled ABO serum control with a mean fluorescence peak at channel  $48 \pm 8$  (mean  $\pm$  SD). Because control sera demonstrated moderate nonspecific Ig binding to granulocytes, patient's sera were compared with the ABO pooled control sera in all assays. A, B, O, and AB sera were also compared individually. Anti-neutrophil antibody positivity was defined as a mean peak fluorescence value more than two standard deviations above the mean value for 20 normal serum controls reflecting all serotypes.

**Polyacrylamide gel electrophoresis (PAGE) and immunoblot studies of neutrophil membrane antigens.** Neutrophil membranes were prepared by nitrogen cavitation of whole cells, followed by subcellular fractionation with sucrose density gradient ultracentrifugation.<sup>13</sup> The membrane layer was further concentrated by centrifugation at  $100,000 \times g$  for 2 hours, and the membrane vesicle-rich pellets were stored at  $-70^\circ\text{C}$  until use. Ten percent PAGE was performed under nonreducing conditions following the technique of Laemmli.<sup>15</sup> An aliquot of the neutrophil membrane preparation, containing approximately 1 mg of protein, was delivered to a single broad well on the stacking gel. After electrophoresis and transfer to nitrocellulose paper, the single broad electrophoresis

preparation was cut into 20 to 30 strips, with approximately 30 to 50 mcg of electrophoretically separated protein per strip.

Immunoblotting was performed using an enzyme-linked (alkaline phosphatase) method similar to that described by Towbin et al and Sidberry et al.<sup>16,17</sup> The primary antibodies were: patients' or controls' serum, purified IgG, or purified F(ab')<sub>2</sub> preparations; rabbit anti-actin antiserum (ICN Immunobiologicals, Naperville IL; immunogen: chicken back muscle actin); murine monoclonal anti-actin IgG or anti-actin IgM (gift of Dr James Lessard, University of Cincinnati, OH; MoAbs C4 [MslgG] and 56-4 [MslgM], immunogen: chicken gizzard actin). The secondary antibodies were: goat anti-human IgG, heavy and light chain specific, alkaline phosphatase conjugated (Cappel Laboratories, Cooper Biomedical Inc); goat anti-rabbit IgG, alkaline phosphatase conjugated (ICN Immunobiologicals); goat anti-mouse Igs, alkaline phosphatase conjugated (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Additional immunoblot studies, under identical conditions, were performed using purified *Acanthamoeba* actin (gift of Dr Blair Bowers, National Institutes of Health, Bethesda, MD).

**IgG preparations and F(ab')<sub>2</sub> fragments.** Purified IgG was prepared from human serum by affinity chromatography with protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ). F(ab')<sub>2</sub> fragments were prepared by pepsin digestion, and separated by affinity chromatography.<sup>18,19</sup>

**Affinity isolation of human anti-actin Ig and detection of anti-actin antibodies by enzyme-linked immunosorbent assay (ELISA).** Sepharose-actin affinity resin was prepared following the method of Craig et al.<sup>20</sup> Patient's serum or purified Ig was passed over the column twice, and specific anti-actin Ig was collected as described.<sup>20</sup>

ELISA of anti-actin specific antibodies was done following the method of Mejean et al,<sup>21</sup> with casein-thimerosal as a blocking agent.<sup>22</sup>

## RESULTS

**Identification of neutropenic patients with anti-neutrophil antibodies.** The sera of 70 patients with suspected autoimmune neutropenia were screened for evidence of anti-neutrophil antibodies. Inclusion criteria for study included an absolute neutrophil count of 1,500 or less, and the absence of another reasonable explanation for neutropenia, such as cytotoxic chemotherapy or malignancy involving the bone marrow. Specifically included were patients with neutropenia and conditions known to be associated with anti-neutrophil antibodies, including seropositive rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), immune thrombocytopenic purpura (ITP), Coombs'-positive hemolytic anemia, lymphoproliferative disease, and chronic hepatitis. Additionally included were patients with neutropenias associated with certain drugs such as penicillins, cephalosporins, phenothiazines, antithyroid drugs, antiarrhythmic drugs (quinidine, procainamide), and phenytoin. Twenty normal healthy laboratory personnel and 22 non-neutropenic patients with rheumatoid arthritis were donors of control sera. None of these control sera had anti-neutrophil antibody activity by either the immunofluorescence or radiometric assays (see Materials and Methods). Moreover, no correlations between ABO serotypes or rheumatoid factor positivity and the degree of nonspecific antibody binding to neutrophils were observed. On the other hand, sera from 36 of the neutropenic patients were found to be positive for anti-neutrophil antibodies by these assays (Table 1). This clinically heterogeneous population of patients with neutropenia

Table 1. Patients With Neutropenia and Anti-Neutrophil Antibodies

Diagnostic Group	Total "Positive" * Anti-Neutrophil Antibodies		"Strongly Positive" * Anti-Neutrophil Antibodies	
	N	ANC† Mean (Range)	N	% of Total
Rheumatoid arthritis	9	870 (40-1,500)	5	56%
Other autoimmune‡	5	660 (100-1,340)	4	80%
Chronic idiopathic	10	460 (0-1,160)	3	30%
Drug-related§	4	260 (0-600)	2	50%
Other	8	900 (160-1,500)	3	38%
Total	36	665 (0-1,500)	17	47%

\*"Positive" defined as greater than 2 standard deviations (SD) above the mean value for IgG binding observed with 20 normal serum controls; "strongly positive" defined as greater than 4 SD above the mean of control values.

†ANC, absolute neutrophil count at the time of study;  $\times 10^6/L$ .

‡ITP (1), SLE (2), mixed connective tissue disease (1), Sicca syndrome (1).

§Trimethoprim-sulfamethoxazole (1), penicillin (1), procainamide (2).

||Chronic hepatitis (2), HIV disease (2), malignancy (2), postviral (2).

and anti-neutrophil antibodies had associated diagnoses of RA, SLE, ITP, other autoimmune disease, malignancy, viral infections, human immunodeficiency virus (HIV) disease, and chronic hepatitis. Anti-neutrophil antibodies in four patients were associated with drugs. Chronic neutropenia not associated with a known underlying disorder or drugs was present in 10 patients (6 adults, 3 children, and 1 neonate). In this group of patients, strongly positive anti-neutrophil antibody activities were observed most frequently in sera from patients with RA and other autoimmune diseases or with apparent drug reactions (Table 1).

*Specific binding of IgG from anti-neutrophil antibody positive sera to neutrophil membrane-associated proteins.* The 36 sera positive for anti-neutrophil antibodies were evaluated for specific binding of IgG to electrophoretically separated neutrophil membrane-associated proteins using SDS-PAGE under nonreducing conditions and immunoblot techniques. Various patterns of IgG binding to membrane-associated proteins were detected with these sera, as illustrated in Fig 1.

All immunoblots from neutrophil membrane preparations had a densely labeled band at approximately 150 Kd (not visible in Fig 1 because it lies above the molecular weight [mol wt] range shown). This band was present both with and without the addition of test sera to the blots, and hence likely represented IgG present in neutrophil membrane preparations. Sera and IgG preparations from both patients and controls also frequently identified a faint broad band extending from about 50 to 70 Kd. This band was absent from all F(ab')<sub>2</sub> blots (see below), and may have represented binding of IgG to neutrophil Fc receptors.<sup>23</sup>

Although a variety of banding patterns emerged from immunoblots with patients' sera, there was no single antigen identified by a majority of sera using this technique. Nonetheless, a 43-Kd neutrophil membrane-associated protein was consistently identified by 8 of the 36 sera positive for anti-neutrophil antibodies. This binding, which was of variable intensity among these sera as illustrated in Fig 1 (lanes B and D through G), was not detected in any of the control sera. To confirm the specificity of Ig binding, IgG preparations and F(ab')<sub>2</sub> fragments were made from the sera of four of these patients from whom adequate supplies of serum were

available. Control IgG and F(ab')<sub>2</sub> preparations were made from four anti-neutrophil antibody positive sera that did not bind to this 43-Kd protein, and from four normal control sera. Each of the IgG and F(ab')<sub>2</sub> preparations from the

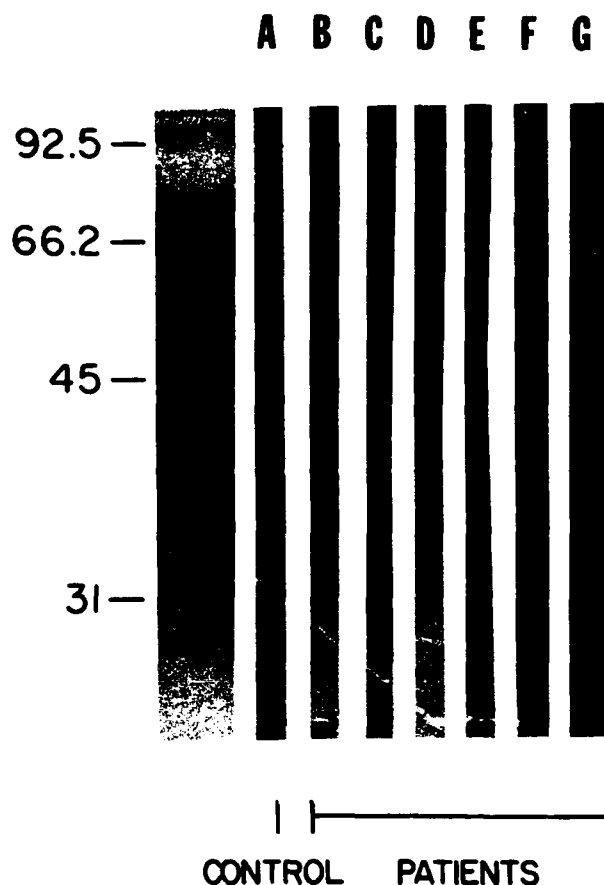


Fig 1. Immunoblot banding patterns in autoimmune neutropenia. The left lane is a coomassie blue stain of neutrophil membrane proteins on nitrocellulose paper. Lane A: Immunoblot of serum from a healthy, normal volunteer. Lanes B through G: Immunoblots of sera from patients with neutropenia and anti-neutrophil antibodies demonstrating various banding patterns.

index patients also identified the 43-Kd protein, while none of the control preparations demonstrated this binding pattern, as illustrated in Fig 2.

**Evidence for anti-actin specificity.** Further studies indicated that actin, which has a mol wt of 43 Kd and is a known constituent of isolated neutrophil membrane preparations,<sup>24</sup> was the protein recognized by these anti-neutrophil antibody positive sera, IgG, and F(ab')<sub>2</sub>. Purified *Acanthamoeba* actin comigrated with the 43-Kd neutrophil membrane-associated antigen when subjected to PAGE. Moreover, sera and purified IgG from the eight patients that recognized the 43-Kd protein also bound specifically to this purified actin in immunoblots, while control sera and IgG, as well as anti-neutrophil antibody positive sera and IgG that did not bind to the 43-Kd protein, did not. The anti-actin binding activity of these sera and purified IgG is illustrated in Fig 3. In addition, a polyclonal anti-actin antiserum prepared in rabbits, as well as two previously defined murine MoAbs with anti-actin specificities (MoAbs C4 [M<sub>1</sub>IgG] and 56-4 [M<sub>1</sub>IgM], see Materials and Methods), were found to bind avidly to a 43-Kd neutrophil membrane-associated protein, which appeared to be the same protein band that was identified by the human anti-neutrophil Igs (illustrated in Fig 3, lane A). When tested by ELISA, the sera that demonstrated binding to the 43-Kd membrane-associated protein also demonstrated anti-actin activity, which was not detectable in control sera. The anti-actin titers were relatively low, in all cases 1:500 or less, compared with the rabbit anti-actin

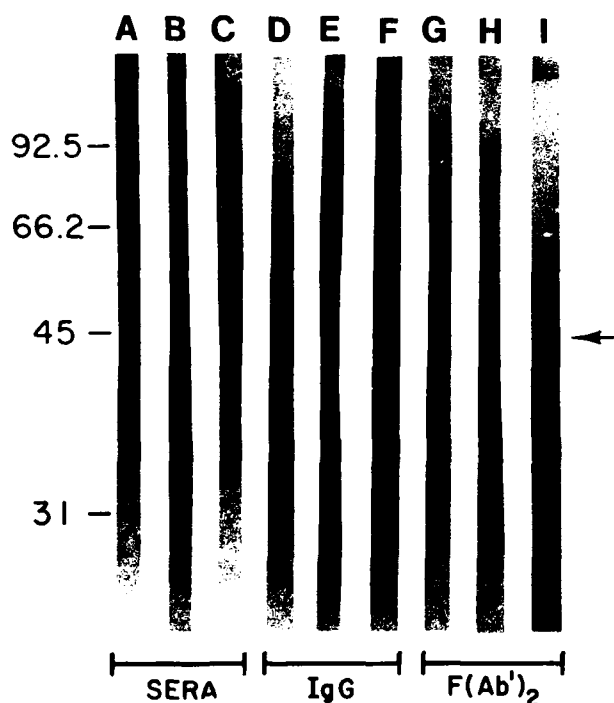


Fig 2. Immunoblot detection of antibody to a neutrophil membrane-associated 43-Kd protein. Neutrophil membranes were electrophoresed and transferred to nitrocellulose paper as described in Materials and Methods. The nitrocellulose was cut into strips and incubated with serum, IgG preparations, or F(ab')<sub>2</sub> preparations. Lanes A, D, and G: control; lanes B, E, and H: patient 4 (Table 2); lanes C, F, and I: patient 1 (Table 2).

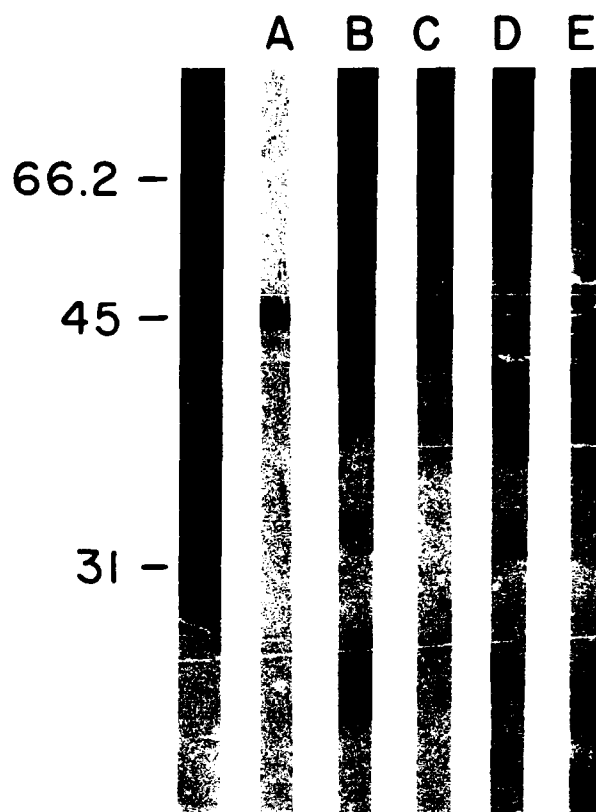


Fig 3. Apparent identity of the 43-Kd neutrophil membrane-associated protein as actin. The left lane is a coomassie blue stain of *Acanthamoeba* actin on nitrocellulose paper. Lane A illustrates binding of monoclonal anti-actin IgG (C4) to blotted neutrophil membrane proteins. The reactive band comigrates with purified actin, and appears to be the same band labeled by patients' IgG as in Fig 2. Lanes B and C are immunoblots of *Acanthamoeba* actin incubated with serum and purified IgG, respectively, from patients 4 and 2, illustrating binding of patients' IgG to actin. Lanes D and E illustrate absence of binding by purified IgG from two controls to actin.

antiserum that was positive at >1:1,600. Nonetheless, sera that showed the most intense binding to the 43-Kd membrane-associated protein in immunoblots had, in general, the highest titers of anti-actin activity by ELISA.

**Binding of anti-actin Igs to intact human neutrophils.** To evaluate the possibility that anti-actin antibodies might contribute to the anti-neutrophil antibody activities detected in patients' sera, we evaluated the binding of known anti-actin Igs (described above) to intact human neutrophils in the immunofluorescence anti-neutrophil antibody assay. The murine monoclonal IgG and IgM antibodies, when tested with appropriate murine isotype controls, did not exhibit specific neutrophil binding. However, the rabbit polyclonal anti-actin antiserum exhibited substantial anti-neutrophil antibody activity when compared with nonimmune rabbit serum controls. This finding is illustrated in Fig 4B, and is compared with the enhanced IgG binding of a patient's serum that was positive for anti-neutrophil antibodies in Fig 4A.

In addition, a human anti-actin Ig was prepared from the serum of one of the index patients (patient no. 3 in Table 2)

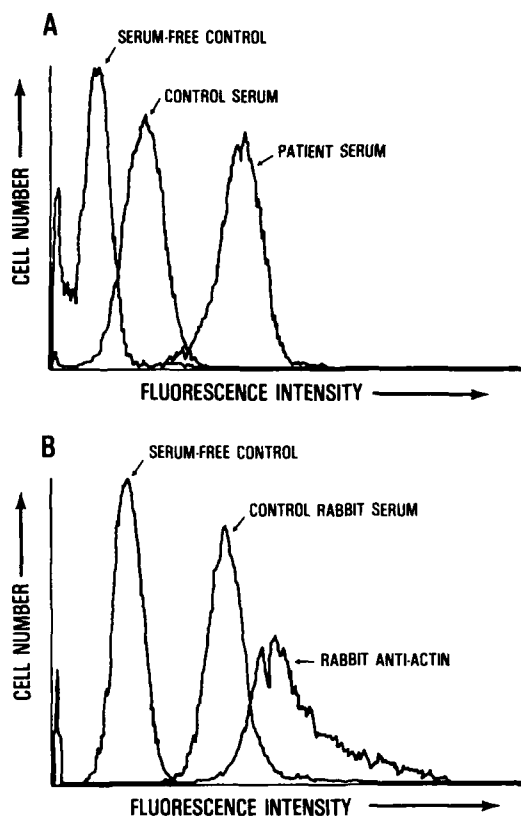


Fig 4. Immunofluorescence assay with intact human neutrophils, demonstrating anti-neutrophil antibody reactivity in serum of a patient with neutropenia and in a rabbit anti-actin antiserum. Normal human neutrophils were incubated first with serum and then with FITC-labeled anti-IgG. Fluorescence was analyzed by flow cytometry, as described in Materials and Methods. (A) Serum from patient 4 (Table 2), normal pooled control serum, and serum-free control. (B) Rabbit anti-actin antiserum, rabbit serum control, and serum-free control.

using a Sepharose-actin affinity column. This human anti-actin also exhibited strong anti-neutrophil antibody binding in the immunofluorescence assay. Furthermore, the increased IgG-specific fluorescence of neutrophils incubated

with this anti-actin IgG preparation was as great as that of cells incubated with the patient's unseparated IgG, as illustrated in Fig 5A, while the IgG from the patient that did not bind to the actin affinity column was devoid of anti-neutrophil antibody binding (Fig 5B).

**Clinical characteristics of patients with apparent anti-actin autoantibodies and neutropenia.** Clinical characteristics of the eight patients with autoimmune neutropenia and apparent anti-actin specific antibodies are listed in Table 2. Of the 8 patients, 2 had rheumatoid factor positivity (patients 1 and 4, Table 2) and 1 had anti-nuclear antibody (patient 5). The absolute neutrophil counts (ANCs) for the eight anti-actin positive patients at the time of serum collection had a range of 0 to 1,500, with a median value of 255 and a mean value of 574. The ANCs for the remaining patients with autoimmune neutropenia, but without anti-actin specificity, also had a range of 0 to 1,500, but the median and mean ANC values (650 and 721, respectively) were somewhat higher. The majority of patients (7 of 8) with specific IgG binding to the 43-Kd membrane-associated protein had "strongly positive" anti-neutrophil antibody reactive sera (Table 3).

When all anti-neutrophil antibody positive sera were considered in relation to degree of neutropenia (Table 3), there was a statistically significant correlation by chi-square analysis between strongly positive anti-neutrophil reactivity ( $>4$  SD  $\nu$  2 to 4 SD above mean control values) and the severity of neutropenia (ANC = 0 to 500  $\nu$  ANC = 500 to 1,500),  $P < .02$ . When this group of anti-neutrophil antibody positive sera were further subdivided into those with (Table 3A) and without (Table 3B) apparent anti-actin specificity, both subgroups showed a trend toward a positive correlation between the strength of anti-neutrophil reactivity and severity of neutropenia ( $P = .17$  and  $.08$ , respectively).

## DISCUSSION

Using an immunoblot technique applied to electrophoretically separated human neutrophil membrane-associated proteins, we detected the presence of specific antibody binding to a 43-Kd protein that appears to be actin in the sera of 36 patients with autoimmune neutropenia. This antibody activ-

Table 2. Patients With Autoimmune Neutropenia and Antibody to Actin

Patient	Age/Race, Sex	Associated Diagnoses	ANC*
1	43/WF	Felty's syndrome	1,500
2	40/BM	HIV antibody, lymphadenopathy, dementia	1,100
3	46/WF	Repeated bacterial infections	200
4	70/WF	History of rheumatoid arthritis, repeated bacterial infections, large granular lymphocytes	40
5	69/WF	Mixed connective tissue disease, chronic candida pyelonephritis, gram-negative sepsis (died)	1,340
6	5 mo/BF	Multiple congenital anomalies, repeated bacterial infections (died)	0
7	41/WF	Sicca syndrome with arthralgias	310
8	16/WF	Immune thrombocytopenic purpura, repeated bacterial infections	100

\*ANC, absolute neutrophil count at the time of study  $\times 10^6/L$ .

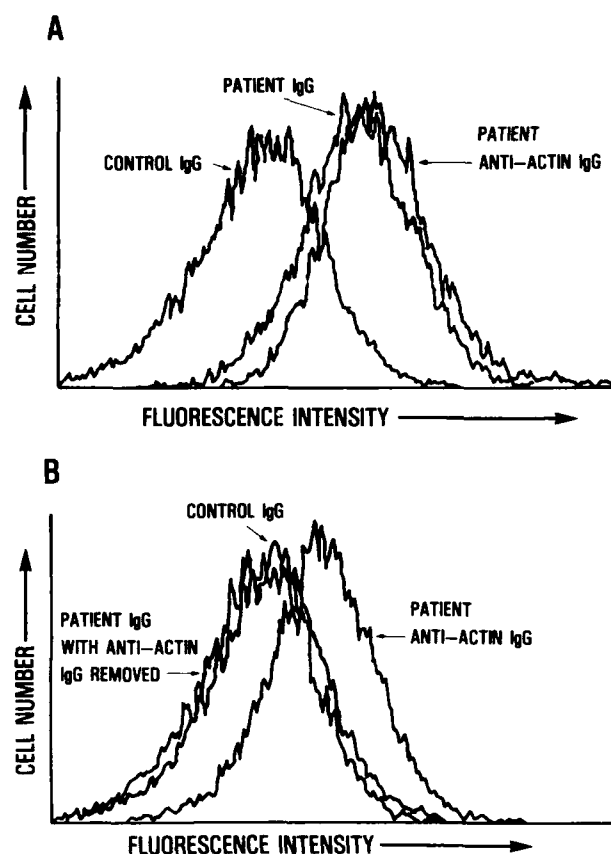


Fig 5. Purified IgG demonstrating anti-neutrophil antibody reactivity in the immunofluorescence assay with intact human neutrophils. (A) Purified IgG from patient 3 (Table 2) exhibits enhanced binding to neutrophils compared with pooled control IgG; additionally, affinity-purified anti-actin IgG from the same patient retains this anti-neutrophil reactivity. (B) Purified IgG from patient 3 exhibits a decrease in anti-neutrophil reactivity, after passage over a Sepharose-actin affinity column (see Materials and Methods). Concentrations of IgG were equivalent in all assays.

ity was not detected in the sera of 20 normal controls nor 22 non-neutropenic patients with seropositive rheumatoid arthritis. Moreover, the apparent anti-actin antibodies detected in these patients were shown to bind to the membrane-associated protein via their  $F(ab')_2$  portions, indicating antigen-specific binding. In addition, we observed that both a polyclonal rabbit anti-actin antiserum and an affinity-purified human anti-actin IgG expressed anti-neutrophil antibody reactivity in assays using intact neutrophils. Hence, these studies indicate that certain anti-neutrophil antibodies detected in autoimmune neutropenia recognize an antigenic epitope of actin.

It has been suggested that actin and Ig may interact in a manner independent of antigenic specificity.<sup>18,25</sup> Such interactions have been reported to be unrelated to antigenic specificity of Ig, of low affinity, and mediated by the Fc portion of the Ig molecule. However, in our hands the binding of IgG to the 43-Kd membrane-associated protein that was detected was found to represent a specific antigen-antibody interaction. Using similar concentrations of sera, IgG, and  $F(ab')_2$ , binding to the apparent actin band was consistently positive with Ig from index patients, but was negative in all controls tested.

Antibodies to actin are well-described as the major component of smooth muscle autoantibodies (microfilament antibodies), with specificity for actin being most common in chronic active hepatitis.<sup>26,27</sup> Anti-actin antibodies have also been detected in various other autoimmune disorders including SLE, Sjögren's syndrome, and myasthenia gravis, as well as in primary biliary cirrhosis and alcoholic liver disease.<sup>27-29</sup> Guilbert et al<sup>30</sup> have reported that anti-actin autoantibodies may be isolated from pooled sera of healthy individuals using affinity chromatography, and these findings suggest that low titer anti-actin autoantibodies may be present in some normal individuals. Whether the presence of anti-actins in certain disease states represents a generalized response to circulating free actin released during cell injury, or a more specific immune response relevant to the pathogenesis of diseases in which these autoantibodies are observed, is unclear.

Table 3. Correlation of Neutropenia With Anti-Neutrophil Antibody Positivity

Degree of Neutropenia*	Degree of Anti-Neutrophil Antibody Positivity†			Totals
	2-3 SD	3-4 SD	> 4 SD	
A. Anti-neutrophil antibody positive patients with anti-actin reactivities				
Mild	1	0	2	3
Moderate	0	0	0	0
Severe	0	0	5	5
Totals	1	0	7	8
B. Anti-neutrophil antibody positive patients without anti-actin reactivities				
Mild	5	1	0	6
Moderate	3	1	3	7
Severe	3	1	6	10
Totals	11	3	9	23

All study patients had ANC's  $\leq 1,500$ ; however, the precise counts for ANC at the time of serum collection were available for only 31 of 36 study patients, 8 with and 23 without anti-actin reactivities.

\*Defined as mild (ANC = 1,000 to 1,500), moderate (ANC = 500 to 1,000), severe (ANC = 0 to 500). ANC, absolute neutrophil count  $\times 10^6/L$ .

†Anti-neutrophil antibody positivity was defined as IgG binding to normal, intact neutrophils that was greater than 2 standard deviations (SD) more than the mean for 20 normal serum controls; degrees of positivity are expressed as SDs greater than the mean of control values.

Our present report is not the first to describe the presence of autoantibodies against a membrane-associated protein of approximately 43 Kd in patients with an immune hematologic disorder. In a patient with chronic ITP, Mason and McMillan<sup>31</sup> detected antibody to a 45-Kd platelet antigen using an immunoblot technique similar to our own. Moreover, Sakaguchi et al<sup>32</sup> identified an autoantibody to a 45-Kd neutrophil antigen in the serum of a 50-year-old man with autoimmune neutropenia using an immunoprecipitation technique.

Actin, or molecules with homologous antigenic epitopes, have been detected on the surfaces of lymphocytes,<sup>33,34</sup> and antigenic cross-reactivity and sequence homology between actin and Thy 1.2 has been documented.<sup>35</sup> Moreover, several studies suggested that actin-like membrane proteins may be involved in mediating functional responses of lymphocytes to anti-actin antibodies.<sup>36-42</sup> Additionally, actin-like molecules have been reported to become exposed on the surface of platelets after thrombin-induced activation and granule secretion.<sup>43,44</sup>

In our present studies, we demonstrated anti-actin binding activity against intact neutrophils with both a rabbit anti-actin antiserum and affinity purified human anti-actin IgG. Thus, actin or molecules with actin-like epitopes appear to be expressed on the surface of neutrophils as well. However, it should be emphasized that the molecule or molecules on the surface of intact neutrophils recognized by the polyclonal rabbit and human anti-actin IgGs may not be the same as the 43-Kd membrane-associated protein recovered from disrupted cells, which is labeled in immunoblots and comigrates with cytoplasmic actin. The actin-specific MoAbs, which were found to bind avidly to the 43-Kd membrane-associated protein in immunoblots, did not demonstrate enhanced binding to intact neutrophils. Hence, the actin epitopes recognized by these MoAbs are either not exposed on the cell surface or are not shared by the cell surface molecules

recognized by the polyclonal anti-actins. A similar discordance in the binding of monoclonal and polyclonal anti-actin antibodies to cell surfaces of lymphocytes has been observed.<sup>33</sup>

The eight patients with apparent anti-actin antibodies and chronic neutropenia were of diverse ages and associated clinical conditions. However, as a group their sera shared strongly positive anti-neutrophil antibody reactivity. Five of these eight patients had profound neutropenia, and two died with infectious complications. These were the only deaths in our series. Hence, a possible association of anti-actin antibodies with an increased likelihood of neutropenia-associated infections deserves further study.

It is likely that some if not most membrane-associated antigens recognized by anti-neutrophil antibodies are not detected by the immunoblot technique. It is also likely that anti-actin antibodies constitute only one of many types of such antibodies, and that autoantibodies with separate antigenic specificities may be of greater importance in autoimmune neutropenias. Nonetheless, our findings indicate that autoantibodies with apparent anti-actin specificities are present in the sera of  $\geq 20\%$  of patients with autoimmune neutropenia, and these antibodies may contribute to the anti-neutrophil reactivity of these sera.

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